Localization of Fibronectin Within the Renal Glomerulus and Its Production by Cultured Glomerular Cells

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Fibronectin was shown in the kidney glomerulus by the use of the peroxidase-labeled antibody technique. At the ultrastructural level, fibronectin was present in greatest quantities along those cell surfaces that abut the capillary basement membrane, especially along the capillary epithelial cell foot process. Intracellular staining was also seen in the glomerulus, most notably in the mesangial cell. Fibronectin was present extracellularly in large amounts in glomerular cell culture and was also demonstrated on the cell membrane and intracellularly. (Am J Pathol 96:651–662, 1979)

FIBRONECTIN IS A glycoprotein composed of high molecular weight subunits and found in extracellular fluids and connective tissues of vertebrates.[1,2] On immunofluorescence microscopy, tissue fibronectin is distributed widely in primitive mesenchyme and basement membranes of embryos[3,4]; in adult human tissues, fibronectin localizes to basement membranes and interstitial connective tissue in a pattern similar to reticulum fibers.[5] Large amounts of fibronectin are synthesized by fibroblasts, astroglial cells, and endothelial cells cultured in serum-containing medium, and fibronectin is a major, surface-associated protein of these cultured cells.[6-9]

The present study is concerned with the distribution of fibronectin within the renal glomerulus. Although immunofluorescence microscopy demonstrates fibronectin within glomerular loops,[3-5,10] the precise tissue localization of fibronectin is not known. Findings of synthesis of fibronectin by endothelial cells [8,9] and by several cell types cultured from embryonic kidney [11] suggest that several cell types within the glomerulus can produce fibronectin. Our studies of cultured glomerular cells demonstrate that these cells do indeed produce fibronectin. Precise immunocytochemical localization of fibronectin within the glomerulus suggests that fibronectin functions as an adhesive between cells and the glomerular basement membrane and/or the mesangial matrix.

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Materials and Methods

Isolation of Fibronectin

Plasma fibronectin was purified by affinity chromatography on gelatin-agarose.[12,13] Fibronectin was eluted with 1 M sodium bromide, pH 5.3,[13] dialyzed against Tris-buffered saline, and concentrated by precipitation with 50% saturated ammonium sulfate. The purified protein preparations were similar in all respects to those previously prepared by a more complicated procedure.[14]

Production of Antifibronectin Antibody

Rabbits were immunized as previously described.[14] Antifibronectin antibody was isolated by affinity chromatography on fibronectin-agarose. Purified fibronectin, 2 mg per gm resin, was coupled to cyanogen-bromide–activated agarose.[15] Antibody was eluted with 0.2 M glycine, pH 2.3.

Production of Fab' Fragments

Affinity-column purified antibody was dialyzed against 0.1 M acetate buffer, pH 4.2; the antibody was digested to $F(ab')_2$ with pepsin.[16] Pepsin was added to the globulin at 1.5% of the weight of the immunoglobulins and digested under sterile conditions in a nitrogen environment for 18 hours at 37 C with very gentle stirring. This mixture was neutralized with 0.2 M sodium hydroxide, dialyzed at 4 C for 18 hours against 0.3 M Tris-HCl buffer, pH 8.2, and then concentrated to 8 mg of $F(ab')_2$ per ml over an Amicon XM50 membrane.

β-Mercaptoethanol was added to the $F(ab')_2$ sample for a final concentration of 0.01 M. This was stirred for one hour at room temperature. Iodoacetic acid (final concentration, 1.85 mg/ml) was added to the reaction mixture and stirred until dissolved; then the mixture was left at 4 C for 18 hours. This was dialyzed for 18 hours against several changes of phosphate-buffered saline and then fractionated on Sephadex G-100 packed in a 1.5 × 60 cm column. The final product, Fab' from the second peak, was concentrated to 5 mg/ml over an Amicon UM10 membrane and stored at −80 F.

An alternative source of Fab' fragments was precipitation of whole immune goat serum with 20% sodium sulfate and isolation of immunoglobulin G by DEAE cellulose chromatography,[17] with Fab' isolated following pepsin digestion as described above. The results obtained were identical to those obtained with affinity-purified antibody.

In both cases, sodium dodecyl sulfate polyacrylamide electrophoresis showed the Fab' fragments to be a single polypeptide band with an approximate molecular weight of 50,000.

Conjugation of Proteins With Horseradish Peroxidase

Antifibronectin antibody or wheat germ agglutinin was conjugated to horseradish peroxidase (HRP) using the periodate procedure of Nakane.[18] The conjugate was separated from free peroxidase by passage through a 2.5 × 35 cm Sephadex G-100 column. Immunoglobulins from nonimmune normal rabbit serum were also conjugated to HRP to serve as a control.

Cell Culture

Guinea pig glomeruli were isolated using a nylon screening technique and cultured as previously described.[19] Human infant glomeruli from surgical specimens were isolated and cultured using a similar technique, while adult human glomeruli from surgical specimens were isolated using nylon screens of larger pore diameter for the final sieving (130 μ).[20,21]

Previous morphologic, biochemical, and histochemical studies have demonstrated that

glomerular cells in culture do not contain contaminating tubular cells or fibroblasts.[20,21] Further, we have provided evidence that the predominant cell in glomerular culture is the capillary epithelial cell.[19] Tubular cells were isolated according to the method of Dechenne et al,[22] in which guinea pig kidney cortex is trypsinized and the resultant cell suspension passed through nylon screens to remove glomeruli. The resulting single cell suspension is composed of tubular cells.

Immunohistochemistry of Kidney Sections and Cultured Cells for Light Microscopy

Guinea pig or human kidney was quick frozen in isopentane and the sections were cut on a cryostat. Kidney sections or cultured cells were fixed in the periodate-lysine-paraformaldehyde fixative (PLP) of McLean and Nakane.[23] Other fixatives, including glutaraldehyde and formalin, were found to destroy the antigenicity of kidney sections. It was found that fixation with 1-ethyl-3(3-dimethylamine) propyl carbodiimide HCl (EDC) (0.1% in cacodylate buffer, pH 7.4) gave dramatic staining for HRP–antifibronectin antibody, but tissue preservation was poor. Therefore, the PLP fixative was considered to be the best choice. After washing, the HRP–antifibronectin antibody was placed on top of the section for 120 minutes at 37 C. The diaminobenzidine (DAB)-H_2O_2 reaction was then performed. The slides were mounted in Permount and photographed with a bright-field microscope. Controls included preincubation of fibronectin with antifibronectin antibody or the substitution of HRP-labeled nonimmune immunoglobulins for antifibronectin antibody.

Immunohistochemistry of Kidney Sections and Cultured Cells for Electron Microscopy

A modification of the procedure of Bretton et al was used.[24] Renal cortex of normal guinea pigs was cut into 1-mm pieces, immediately fixed in PLP for 24 hours at 4 C, and then washed for 24 hours in 0.2 M sodium cacodylate buffer (pH 7.4) at 4 C. Several changes of buffer were necessary to prevent subsequent tissue distortion upon freezing. The tissue was then immersed in 10% glycerol in 0.2 M sodium cacodylate buffer (pH 7.4) for 1 hour at room temperature, rinsed with phosphate-buffered saline, embedded in Tissue-Tek, and frozen on dry ice. The tissue was cut in 16-μ sections on an International cryostat and placed on an albuminized slide. Immunohistochemical staining immediately followed sectioning, with incubation of HRP–antifibronectin antibody or HRP–antifibronectin Fab' fragments for 3 hours at room temperature with constant rotary motion. The prolonged incubation and constant agitation were necessary to ensure adequate penetration of antibody molecules. The sections were washed overnight in phosphate-buffered saline at 4 C. The tissue was incubated in DAB (1 mg/ml) in 0.05 M Tris buffer for 20 minutes before the DAB-H_2O_2 reaction was performed. After washing the sections in 0.1 M sodium cacodylate buffer for 1 hour at room temperature, the tissues were postfixed in 2% OsO_4 in 0.1 M sodium cacodylate buffer for 30 minutes, dehydrated with graded acetone 30–100%, and embedded for electron microscopy.

In this paper, we have chosen to counterstain HRP-antifibronectin Fab' stained tissue with uranyl acetate and lead citrate, mainly because unstained sections were especially difficult to interpret. Further, because of the difficulty of penetration of whole antibodies into tissues, we chose to use direct staining with Fab' fragments rather than an indirect procedure in which, although staining would be darker, there would be much more difficulty in penetration through tissues.

Cells on coverslips were fixed with PLP, stained with HRP–antifibronectin antibody, and then processed as whole tissues. Nonimmune rabbit serum conjugated to HRP or preincubation of peroxidase-labeled antifibronectin antibody or HRP–antifibronectin Fab' fragments with purified fibronectin was used as a control for both tissue and cells.

In order to determine better if the staining for fibronectin was unique, lectin staining was also performed. Concanavalin A staining was performed using the two-step procedure described by Bretton et al.[24] Controls were performed by preincubation of concanavalin A

with α-methyl D-mannoside. Tissue sections were also stained with peroxidase-labeled wheat germ agglutinin. Preincubation of the lectin with N-acetyl-D-glucosamine served as a control. Finally, tissue sections were stained with *Ricinus communis* agglutinin, which had been conjugated to horseradish peroxidase.

Results

Immunohistochemistry at the light microscopic level demonstrated large amounts of fibronectin within the renal glomerulus. Both guinea pig and human kidney gave similar results, with glomerular mesangium being strongly positive for fibronectin (Figure 1A). Tubular basement membranes also stained lightly. Controls, including preincubation of fibronectin with antifibronectin antibody (Figure 1B) or the substitution of HRP-conjugated immunoglobulins from nonimmune rabbit serum, had no staining.

To assess the precise localization of fibronectin, electron microscopy was performed on kidney tissue section stained with HRP–antifibronectin antibody. Unfortunately, the morphology seen using whole antibody was not good, with frequent fusion of epithelial cell foot processes. However, the use of HRP-Fab′ fragments avoided these ultrastructural changes. Using HRP-Fab′ antifibronectin, the darkest staining for fibronectin in the guinea pig glomerulus was present along the epithelial cell foot processes, although the endothelial cell membranes were lightly stained along the cell membrane bordering the glomerular basement membrane (Figure 2). Thus, the highest concentration of fibronectin was at the interface between basement membrane and cell (epithelial or endothelial). Sections not counterstained with lead citrate and uranyl acetate clearly demonstrated that the basement membrane and mesangial matrix did not stain at all. In sections fixed with 0.1% EDC, the staining at the interface between basement membrane and cell was dramatic, but unfortunately this compound does not preserve tissue well. The mesangial cell cytoplasm stained more darkly than either epithelial or endothelial cells. Renal tubular cells (proximal or distal tubules) did not stain at all, but occasionally the tubular cell membrane stained lightly. The staining described above in the guinea pig kidney was identical to that found in the human kidney. Controls for electron microscopy involved preincubation of HRP–antifibronectin Fab′ fragments with purified fibronectin; this treatment almost completely abolished the staining reaction normally seen with HRP–antifibronectin Fab′ fragments.

In order to show that this reaction was specific, the distribution of concanavalin A and wheat germ agglutinin receptors was studied within the guinea pig kidney. Concanavalin A stained both basement membrane and epithelial cell foot processes, while wheat germ agglutinin stained

only the glomerular cell membranes (epithelial, endothelial, and mesangial) but not the basement membrane. Both patterns were quite different from fibronectin staining. Control sections in which lectins were preincubated with their specific carbohydrates did not show staining. Staining with *Ricinus communis* agglutinin was identical to fibronectin staining.

Large amounts of fibronectin could be localized to both guinea pig and human glomerular cells in culture when the cells reached confluency. Although some glomerular cells did not stain for fibronectin, the majority had dense strands of fibronectin present in extracellular material (Figures 3A, 3B, and 4) and a granular distribution of fibronectin in the perinuclear region (Figures 3A and 3B). Tubular cells in culture showed no staining. The intracellular membranes of glomerular cells, including mitochondrial membranes, stained lightly for fibronectin. In addition, there was staining of dense granules, especially at the periphery of the granules (Figure 5). The homogeneous appearance of the contents of the dense granules was similar to the appearance of basement membranes, and the granular contents reacted with concanavalin A.

Discussion

Our light microscopic studies confirm the finding of previous immunofluorescence studies that fibronectin is found predominantly in the mesangial region within the renal glomerulus.[3-5,10] Ultrastructural immunohistochemical examination demonstrated that the largest concentration of fibronectin was found at the junction between epithelial cell and basement membrane, although fibronectin was also present in small quantities between endothelial cell and basement membrane, between mesangial cell and mesangial matrix, and between tubular cell and basement membrane. We interpret the mesangial staining seen on light microscopy as staining of the interface between epithelial cell and mesangial matrix and of the mesangial cells themselves. This finding is in agreement with experiments which demonstrate that fibronectin mediates the attachment of cultured cells to collagen [25-27] and with the finding that isolated glomerular basement membranes contain undetectable fibronectin.[28]

Glomerular cells in culture had plentiful extracellular fibronectin. This production in culture may serve as a model for the production of fibronectin by glomerular cells *in vivo*. It is of interest that large quantities of basement membrane (as assessed by HRP-labeled antiglomerular basement membrane antibody from patients with Goodpasture's syndrome) and fibronectin seemed to be produced at similar times in culture, ie, when the cells reach confluency (unpublished observations). The majority

of cells in glomerular culture were positive for fibronectin, although some were negative. Previous studies in our laboratory demonstrated that several glomerular cell types had cell surface fibronectin (most probably epithelial and mesangial cell), while tubular cells did not have cell surface fibronectin.[19]

The nature of the granules seen within cultured glomerular cells has not been proved conclusively. The ultrastructural appearance of the granules and the fact that granular contents reacted with concanavalin A, which we and others [24] have shown to react with glomerular basement membrane, suggest that these granules may be basement membrane. The outer coats of these granules stained for fibronectin, suggesting that the molecules within the granule and fibronectin may be synthesized together in glomerular cells. Intracellular membranes in glomerular cells *in vitro* also stained lightly and specifically with HRP-labeled antifibronectin antibody. It is not clear whether this represents the presence of fibronectin in these membranes or the cross reactivity of the antibody with an integral membrane compound.

By immunofluorescence, Sheinman et al [10] observed increased amounts of fibroblast surface antigen, which is probably identical to fibronectin, in certain glomerular diseases. The observation that fibronectin may promote clumping of platelets and polymorphonuclear leukocytes on arteriovenous shunts [29] raises the possibility that fibronectin could cause similar events *in vivo*. The diverse activities of fibronectin on both inflammatory cells and mesenchymal cells in culture suggest that fibronectin may play a role in many pathophysiologic processes. A combination of immunocytochemical studies of diseased kidneys and studies of the glomerular cells in culture should allow definitive investigations of the role of fibronectin in glomerular diseases.

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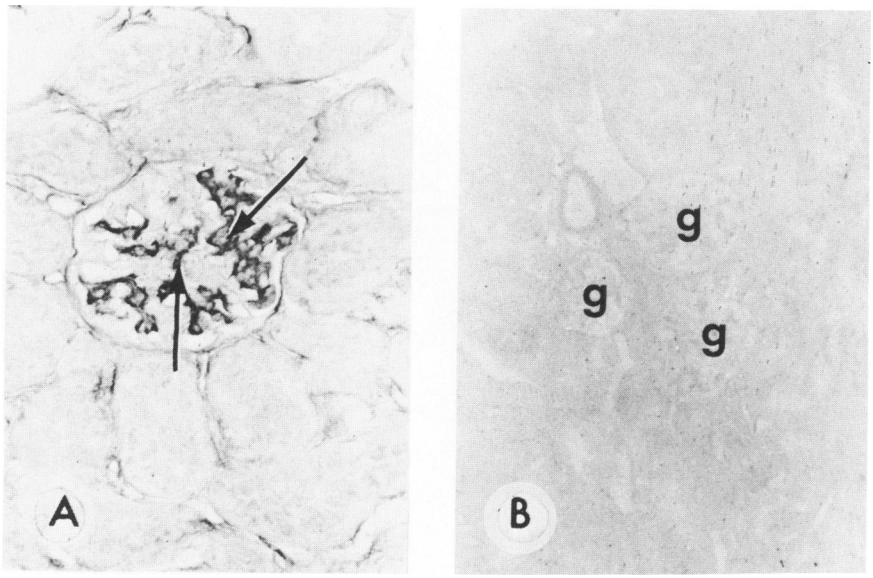


Figure 1A—Light microscopy of guinea pig kidney stained by horseradish-peroxidase (HRP)-labeled antifibronectin antibody. The mesangium appears most strongly stained (*arrows*), but Bowman's capsule membrane and tubular basement membrane also stained lightly. (×324) **B**—Light microscopy of control kidney section in which HRP-labeled antibody was preincubated with purified fibronectin. No staining of glomeruli (*g*) is seen. (×240)

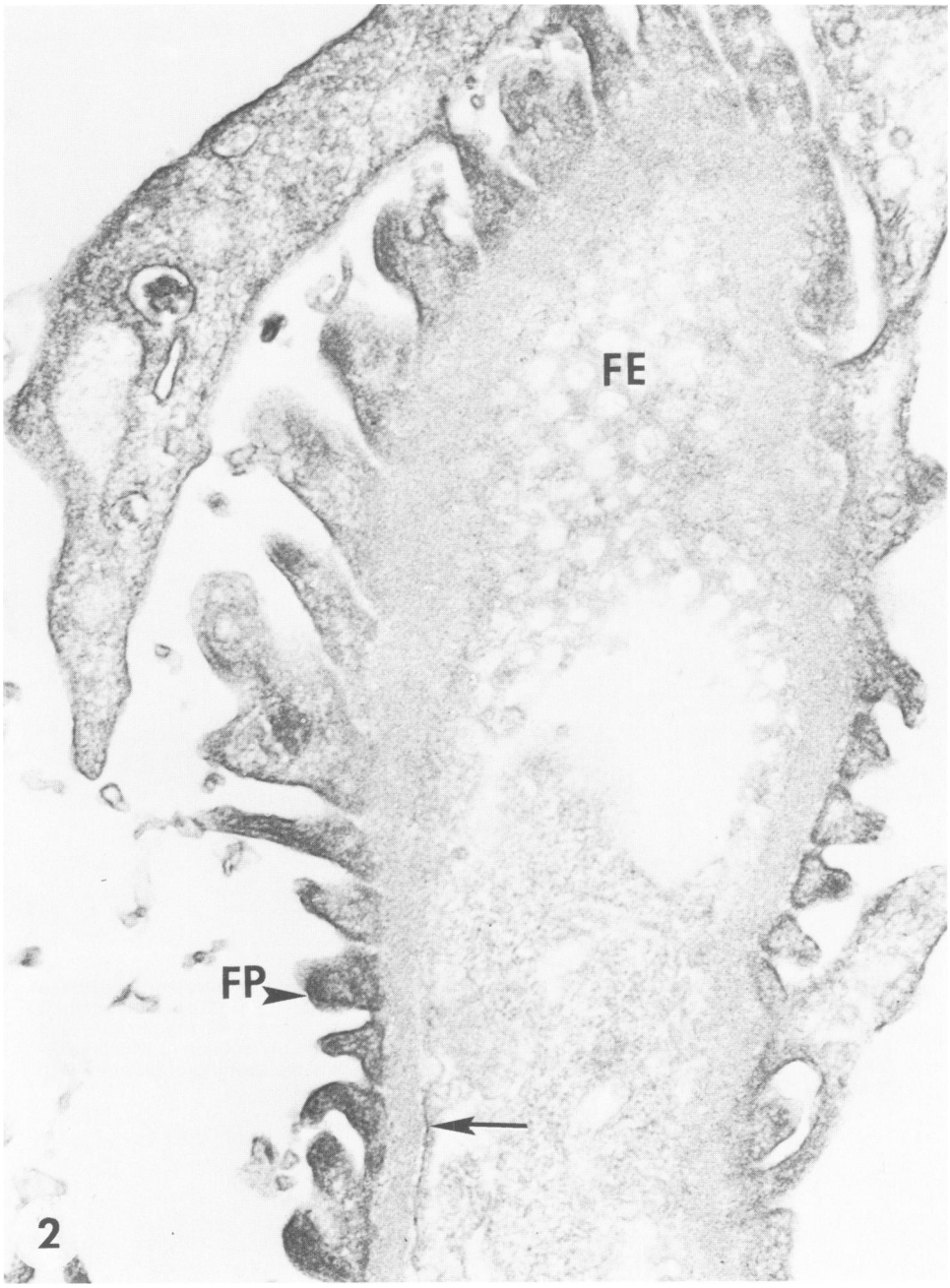
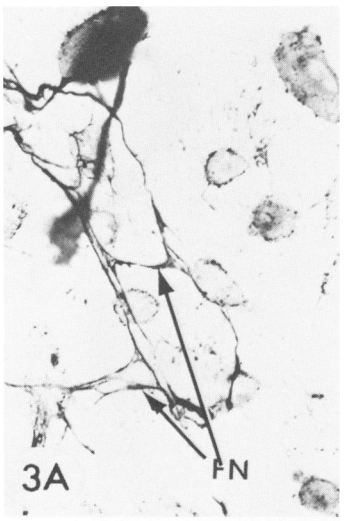
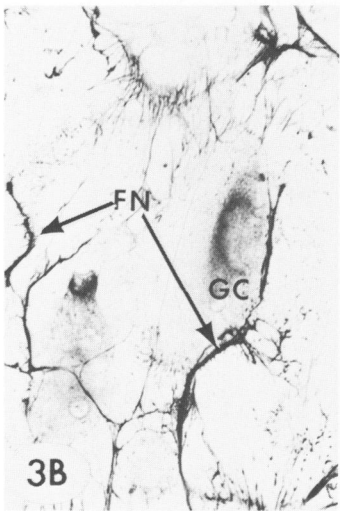


Figure 2—Electron microscopy of guinea pig kidney section stained with HRP-antifibronectin Fab' fragments. Epithelial cell foot processes show dark staining (*FP*), while endothelial cell fenestrae (*FE*) do not stain. In other areas, endothelial cell membrane stains lightly (*arrow*). Even though the section was counterstained with uranyl acetate and lead citrate, it was quite easy to discern the difference in staining between the epithelial cell foot processes and the endothelial cell fenestrae. (×24,233)





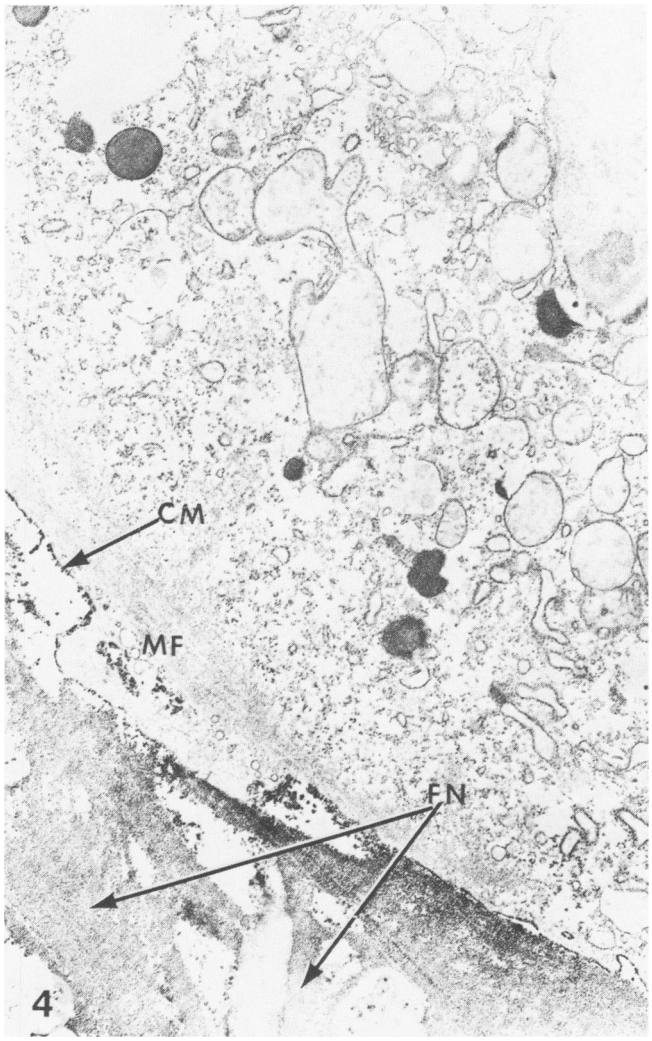


Figure 3A—Light microscopy of human infant glomerular cells *in vitro* stained with HRP-labeled antifibronectin antibody. Coarse strands of extracellular fibronectin (*FN*) are present (*arrows*), and cells show perinuclear staining. (×960) **B**—Light microscopy of human infant glomerular cells *in vitro* stained with HRP-labeled antifibronectin antibody. Coarse strands of extracellular fibronectin (*FN*) are present (*arrows*), and one cell (*GC*) appears to show some cell surface staining. (×960) **Figure 4**—Electron microscopy of guinea pig glomerular cell *in vitro* stained with HRP-labeled antifibronectin antibody. Coarse strands of extracellular fibronectin are present (*FN*). Cell membrane (*CM*) staining is apparent (*arrow*). Intracellular staining of cell membranes is also seen. The section was not stained with either lead citrate or uranyl acetate. *MF* = microfilaments. (×12,540) (Both with a photographic reduction of 13%)

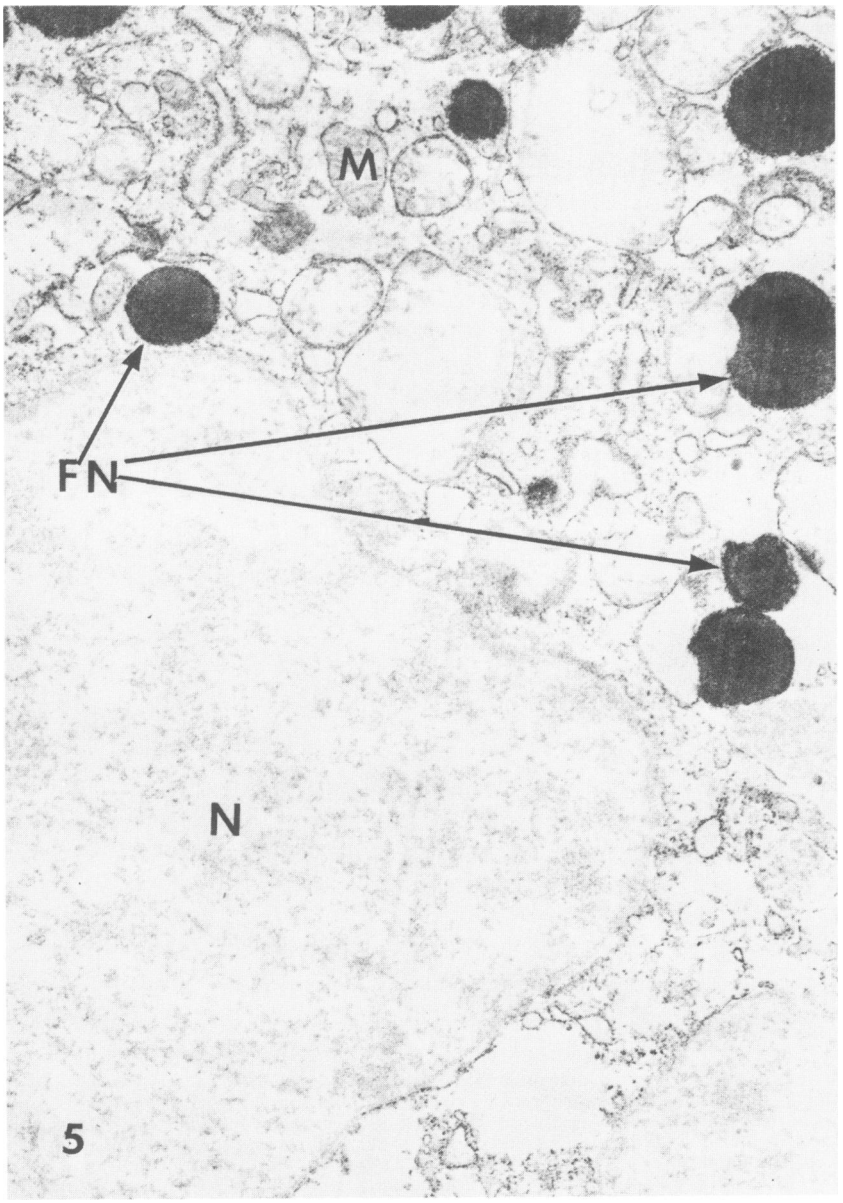


Figure 5—Electron microscopy of guinea pig glomerular cell *in vitro* stained with HRP-labeled antifibronectin antibody. The outer coat of the granules seen within glomerular cells stain especially strongly for fibronectin (*FN*). The section was not stained with either lead citrate or uranyl acetate. *N* = nucleus; M = mitochondrion. (×16,770)